Expression and crystallographic studies of D-glycero- β -D-manno-heptose-1-phosphate adenylyltransferase from *Burkholderia pseudomallei*

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The Gram-negative bacterium *Burkholderia pseudomallei* is the causative agent of melioidosis. D-glycero- β -D-manno-Heptose-1-phosphate adenylyltransferase (HldC) is the fourth enzyme of the ADP-L-glycero- β -D-manno-heptose biosynthesis pathway, which produces an essential carbohydrate comprising the inner core of lipopolysaccharide. Therefore, HldC is a potential target of antibiotics against melioidosis. In this study, HldC from *B. pseudomallei* has been cloned, expressed, purified and crystallized. Synchrotron X-ray data from a selenomethionine-substituted HldC crystal were also collected to 2.8 Å resolution. The crystal belonged to the primitive triclinic space group *P*1, with unit-cell parameters $a = 74.0$, $b = 74.0$, $c = 74.9$ Å, $\alpha = 108.4$, $\beta = 108.4$, $\gamma = 108.0^\circ$. Eight protomers are present in the unit cell and three out of five selenomethionines were found in each protomer using the *PHENIX* software suite. A full structural determination is in progress to elucidate the structure–function relationship of the protein.

1. Introduction

Melioidosis is an infectious disease that is commonly found in Northern Australia and Southeast Asia (Currie *et al.*, 2008). It is the third highest cause of death from infectious diseases in northeast Thailand (Limmathurotsakul *et al.*, 2010) and the fifth in Singapore (Liu *et al.*, 2015). The Gram-negative bacterium *Burkholderia pseudomallei*, the causative agent of melioidosis, has been classified as a potential bioterrorism agent owing to its high accessibility, environmental persistence, high mortality and the lack of an available vaccine (Gilad *et al.*, 2007). It also exhibits resistance to various groups of antibiotics, including penicillins, cephalosporins, rifamycins and aminoglycosides (Cheng & Currie, 2005). Therefore, intensive research to understand the biology of *B. pseudomallei* and to develop antibiotics against melioidosis is required.

In most Gram-negative bacteria, lipopolysaccharide (LPS) is a principal component of the outer membrane. LPS is responsible for the structural integrity and strength of the bacterial outer membrane and its impermeability to toxic hydrophobic compounds (Nikaido & Vaara, 1985). LPS consists of three covalently linked structures known as lipid A, a central core oligosaccharide and an O-specific polysaccharide side chain (Wiese *et al.*, 1999). The core oligosaccharide can be subdivided into two regions: an outer core and an inner core. The inner core contains two or three L-glycero-D-manno-heptoses and one to three 3-deoxy-D-manno-oct-2-ulosonic acids (Heinrichs *et al.*, 1999). Mutation of the genes related to the heptose biosynthesis pathway leads

Table 1
Macromolecule-production information.

Source organism	<i>B. pseudomallei</i> K96243
DNA source	gDNA
Forward primer	5'-GGCGGTGGTGGCGCATGCCGCTTCGTTTCG-AACG-3'
Reverse primer	5'-GTTCTTCTCCTTTGCGCCCTAGCGCGACTG-CGCGCG-3'
Cloning vector	LIC expression vector pB2
Expression vector	LIC expression vector pB2
Expression host	<i>E. coli</i> BL21 (DE3) for native <i>BpHldC</i> , <i>E. coli</i> B834 (DE3) for SeMet- <i>BpHldC</i>
Complete amino-acid sequence of the construct produced	MHHHHHGGGGMPASFERKLITRDALAAMRASL-PAPVVFTNGVFDILHRGHVSYLADAKALGACL-IVGVNSDASVRLMGKGGDRPINVQEDRMALLA-ALECVDWVGDFEKTTPVSLIEAVHPDILVKGG-DYMDALPESALVRGWGGRALAIPFEHDSSTT-ALLKKVRAQSR

to loss of virulence and increased sensitivity to antibiotics in some organisms (Harper *et al.*, 2004; Hiratsuka *et al.*, 2005; Kim, 2003) and death in *Pseudomonas aeruginosa* (Walsh *et al.*, 2000). Therefore, the heptose biosynthesis pathway is considered to be an attractive target for new antibiotics.

D-glycero-β-D-manno-Heptose-1-phosphate adenylyltransferase (HldC, formerly RfaE or HldE2) is the fourth enzyme of the biosynthetic pathway for ADP-L-glycero-β-D-manno-heptose (Fig. 1). In many species, the bifunctional enzyme HldE catalyzes the second and fourth steps of the pathway. HldE is composed of an N-terminal kinase domain and a C-terminal adenylyltransferase domain (Kneidinger *et al.*, 2002), which are homologous to D-glycero-D-manno-heptose-7-phosphate kinase (HldA) and HldC, respectively. HldC transfers the AMP moiety from ATP to D-glycero-β-D-manno-heptose-1-phosphate and releases ADP-D-glycero-β-

D-manno-heptose and pyrophosphate. The crystal structure of HldA from *B. cenocepacia* has been determined (Lee *et al.*, 2013), but that of HldC has not yet been reported. To elucidate the structure–function relationship of HldC from *B. pseudomallei* (*BpHldC*), its crystallographic study has been carried out. Here, we report the cloning, overexpression, purification, crystallization and preliminary X-ray study of *BpHldC*.

2. Materials and methods

2.1. Macromolecule production

2.1.1. Cloning of *rfaE* from *B. pseudomallei*. The *rfaE* gene coding for the HldC protein (NCBI Reference Sequence YP_107021.1) was amplified by PCR using 200 ng *B. pseudomallei* genomic DNA template and 50 μM of primers. The primer sequences used for ligation-independent cloning (LIC) are shown in Table 1. PrimeSTAR HS DNA polymerase with GC buffer (Takara Bio Inc., Shiga, Japan) designed for high-GC-content genomic DNA was used. The LIC expression vector pB2 (Kim *et al.*, 2005), which expresses the cloned gene fused to a noncleavable N-terminal His₆ tag, was digested with SmaI. The PCR product treated with T4 DNA polymerase (New England Biolabs, Beverly, Massachusetts, USA) in the presence of dTTP was ligated with the digested vector incubated with T4 DNA polymerase in the presence of dATP. The ligation product was transformed into DH5α competent cells. Clones were screened by plasmid DNA analysis and transformed into *Escherichia coli* BL21 (DE3) cells for the expression of native *BpHldC* and *E. coli* B834 (DE3) cells for the expression of selenomethionine-substituted *BpHldC* (SeMet-*BpHldC*).

Figure 1
Schematic diagram of the ADP-L-glycero-β-D-manno-heptose biosynthesis pathway. Abbreviations are as follows: GmhA, sedoheptulose-7-phosphate isomerase; HldA, D-glycero-β-D-manno-heptose-7-phosphate kinase; GmhB, D-glycero-D-manno-heptose-1,7-bisphosphate phosphatase; HldC, D-glycero-β-D-manno-heptose-1-phosphate adenylyltransferase; HldD, ADP-L-glycero-D-manno-heptose-6-epimerase.

Table 2
Crystallization of SeMet-*BpHldC*.

Method	Hanging-drop vapour diffusion
Plate type	VDX48 plate (Hampton Research)
Temperature (K)	296
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	20 mM Tris-HCl pH 8.0, 0.35 M NaCl, 8.5 mM <i>n</i> -octyl- β -D-thiomaltoside
Composition of reservoir solution	0.1 M HEPES pH 7.0, 20% (w/v) polyethylene glycol 3350
Volume and ratio of drop	1.6 μ l, 1:1
Volume of reservoir (μ l)	200

2.1.2. Protein expression and purification of native *BpHldC*. *E. coli* BL21 (DE3) cells transformed with the cloned vector harbouring the *rfaE* gene were grown on Luria-Bertani (LB) agar plates containing 150 μ g ml⁻¹ ampicillin. Several colonies were picked and grown in capped test tubes with 10 ml LB broth containing 150 μ g ml⁻¹ ampicillin. A cell stock of 0.85 ml culture and 0.15 ml glycerol was prepared and frozen at 193 K for use in a larger culture. The frozen cell stock was grown in 5 ml LB medium and diluted into 1000 ml fresh LB medium. The culture was incubated at 310 K with shaking until an OD₆₀₀ of 0.6–0.8 was reached. At this point, expression of *BpHldC* was induced using isopropyl β -D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. The culture was further grown at 298 K for 16 h in a shaking incubator. Cells were harvested by centrifugation at 7650g (6500 rev min⁻¹) for 10 min in a high-speed refrigerated centrifuge at 277 K. The cultured cell paste was resuspended in 25 ml of a buffer consisting of 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g ml⁻¹ DNase I. The cell suspension was disrupted using a high-pressure homogenizer (Emulsiflex-B15, Avestin, Canada). Cell debris was pelleted by centrifugation at 24 900g (15 000 rev min⁻¹) for 30 min in a high-speed refrigerated ultracentrifuge at 277 K.

The supernatant was affinity-purified using a HisTrap column on an ÄKTAexplorer system (GE Healthcare, Piscataway, New Jersey, USA). The column was equilibrated with a buffer consisting of 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole. The target protein was eluted with a buffer consisting of 50 mM Tris-HCl pH 8.0, 100 mM NaCl with a gradient from 10 to 500 mM imidazole. The protein was further purified by ion-exchange chromatography using a 5 ml HiTrap Q column (GE Healthcare). The column was equilibrated with a buffer consisting of 20 mM Tris-HCl pH 8.5. The protein was eluted at 0.16 M NaCl using a linear NaCl gradient. The purified native HldC protein containing a non-cleavable N-terminal His₆ tag was concentrated to 10 mg ml⁻¹ for crystallization.

2.1.3. Protein expression and purification of SeMet-*BpHldC*. To obtain SeMet-*BpHldC*, a frozen cell stock of *E. coli* B834 (DE3) cells was prepared using the same method as for *E. coli* BL21 (DE3) cells. The frozen cell stock was grown in 5 ml LB medium and diluted into 1000 ml medium containing M9 salts supplemented with 20% (w/v) glucose, 1 M MgSO₄, 1 M CaCl₂, 0.5% (w/v) thiamine, 100 μ g ml⁻¹ of each of arginine, histidine, isoleucine, leucine, glutamate, lysine,

phenylalanine, cysteine and aspartate, 150 μ g ml⁻¹ valine, 400 μ g ml⁻¹ serine, 400 μ g ml⁻¹ threonine, 30 μ g ml⁻¹ tyrosine, 40 μ g ml⁻¹ tryptophan, 50 μ g ml⁻¹ L-selenomethionine and 150 μ g ml⁻¹ ampicillin. Protein expression and purification of SeMet-*BpHldC* was performed using the same protocol as for the native protein. The anion-exchange column was equilibrated with a buffer consisting of 20 mM Tris-HCl pH 8.0. The protein was eluted at 0.35 M NaCl using a linear NaCl gradient. The purified SeMet-*BpHldC* protein containing a noncleavable N-terminal His₆ tag was concentrated to 10 mg ml⁻¹ for crystallization. The purity of native *BpHldC* and SeMet-*BpHldC* was checked on SDS-PAGE. They were also pretreated with 1 mM dithiothreitol (DTT) to run SDS-PAGE gels under reducing conditions.

2.2. Crystallization

Screening for initial crystallization conditions was performed with native *BpHldC*. Screening was carried out at room temperature using the sparse-matrix method (Jancarik & Kim, 1991) with several screens from Hampton Research (Laguna Niguel, California, USA). A Hydra-Plus-One crystallization robot (Matrix Technologies, Hudson, New Hampshire, USA) was used to set up the screens using the sitting-drop vapour-diffusion method in a 96-well Intelli-Plate (Art Robbins Instrument, Salt Lake City, Utah, USA). Sitting drops were made by mixing 0.2 μ l protein solution with 0.2 μ l reservoir solution and were equilibrated over 50 μ l reservoir solution. Crystals of native *BpHldC* was obtained in the condition 0.1 M MES pH 6.5, 15% (w/v) polyethylene glycol 3350. A crystal of SeMet-*BpHldC* also appeared in the same condition. Optimization was performed using the hanging-drop vapour-diffusion method in a VDX48 plate (Hampton Research) by mixing 0.8 μ l protein solution and 0.8 μ l reservoir solution. To obtain high-quality crystals of SeMet-*BpHldC*, detergent screening was carried out using Detergent Screen HT from Hampton Research. Finally, diffraction-quality crystals of SeMet-*BpHldC* were obtained in 0.1 M HEPES pH 7.0, 20% (w/v) polyethylene glycol 3350, 8.5 mM *n*-octyl- β -D-thiomaltoside. A summary of the crystallization information is given in Table 2.

2.3. Data collection and processing

Before being flash-cooled in liquid nitrogen, crystals were soaked in LV CryoOil (MiTeGen, Ithaca, New York, USA) as a cryoprotectant. X-ray diffraction data were collected at a single wavelength on beamline 7A at Pohang Light Source using an ADSC Quantum Q270r detector placed 300 mm from the sample. The data were collected at the peak absorption wavelength (f'') for the selenomethionine-labelled protein ($\lambda = 0.97927$ Å) with inverse-beam geometry owing to rapid crystal decay. The oscillation range per image was 1°, with 1 s exposure. X-ray diffraction data were processed and scaled using *iMosflm* (Battye *et al.*, 2011) and *AIMLESS* in the *CCP4* suite (Winn *et al.*, 2011).

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Pohang Accelerator Laboratory (PAL)
Wavelength (Å)	0.97927
Temperature (K)	100
Detector	ADSC Quantum Q270r
Crystal-to-detector distance (mm)	300
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	1
Space group	<i>P</i> 1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	74.0, 74.0, 74.9
α , β , γ (°)	108.4, 108.4, 108.0
Mosaicity (°)	0.84
Resolution range (Å)	34.94–2.80 (2.95–2.80)
Total No. of reflections	112530 (16961)
No. of unique reflections	30332 (4415)
Completeness (%)	96.8 (97.0)
Multiplicity	3.7 (3.8)
$\langle I/\sigma(I) \rangle$	15.5 (8.6)
R_{meas} (%)	10.3 (16.5)
Overall <i>B</i> factor from Wilson plot (Å ²)	26.6

3. Results and discussion

The yields of purified native *Bp*HldC and SeMet-*Bp*HldC were 100 and 22.6 mg per litre of *E. coli* culture, respectively. After anion-exchange chromatography, native *Bp*HldC and SeMet-*Bp*HldC appeared to be approximately 99% pure, with a prominent protein band at around 19 kDa on SDS-PAGE (Fig. 2). Information on macromolecule production is summarized in Table 1.

At first, screening experiments for initial crystallization conditions were carried out using the native protein. Crystals of the native protein appeared with dimensions of $0.2 \times 0.1 \times 0.01$ mm in 0.1 M MES pH 6.5, 15% (w/v) polyethylene glycol 3350. Crystals of the selenomethionine-labelled protein also appeared in the same condition as the native protein, but their

size was relatively small. To produce larger crystals suitable for X-ray diffraction, the initial crystallization condition was optimized by varying the concentration of the precipitant and the pH of the buffer. Rod-shaped crystals of SeMet-*Bp*HldC were obtained using 0.1 M HEPES pH 7.0, 20% (w/v) polyethylene glycol 3350, but the X-ray diffraction data from these crystals was not good enough for crystal phasing. Therefore, detergent screening was used to attempt to produce better diffraction-quality crystals. Finally, improved crystals of

Figure 3

Crystals of SeMet-*Bp*HldC. These crystals appeared in the condition 0.1 M HEPES pH 7.0, 20% (w/v) PEG 3350, 8.5 mM *n*-octyl- β -D-thiomaltoside. The crystals grew to approximate dimensions of $0.25 \times 0.15 \times 0.02$ mm in a day.

Figure 2

Coomassie-stained SDS-PAGE (12%) of (a) native *Bp*HldC and (b) SeMet-*Bp*HldC. (a) Lane *M*, molecular-mass marker (labelled in kDa); lane 1, native *Bp*HldC; lane 2, native *Bp*HldC under reducing conditions (1 mM DTT). (b) Lane *M*, molecular-mass marker (labelled in kDa); lane 1, SeMet-*Bp*HldC; lane 2, SeMet-*Bp*HldC under reducing conditions (1 mM DTT).

Figure 4

Diffraction pattern of a SeMet-*Bp*HldC crystal.

SeMet-*BpHldC* appeared in the presence of 8.5 mM *n*-octyl- β -D-thiomaltoside and grew to dimensions of $0.25 \times 0.15 \times 0.02$ mm within a day at 296 K (Fig. 3).

Single-wavelength anomalous dispersion (SAD) data were collected at 0.97927 Å from a SeMet-*BpHldC* crystal to 2.8 Å resolution (Fig. 4). The crystal belonged to the primitive triclinic space group *P*1, with unit-cell parameters $a = 74.0$, $b = 74.0$, $c = 74.9$ Å, $\alpha = 108.4$, $\beta = 108.4$, $\gamma = 108.0^\circ$. Based on the Matthews coefficient (Matthews, 1968), the asymmetric unit could contain eight protomers with 45.8% solvent content ($V_M = 3.67$ Å³ Da⁻¹). Details of the data-collection statistics are presented in Table 3. Crystallographic phases for the 2.8 Å resolution selenomethionine data set were obtained by SAD phasing. The substructure location and automatic model building were carried out using *AutoSol* and *AutoBuild* in the *PHENIX* software suite (Adams *et al.*, 2010). A relatively good electron-density map was calculated and an initial model containing eight protomers in the unit cell was obtained after automated model building. In each protomer, three out of five selenomethionines were found.

BpHldC shares relatively low sequence identity (30%) with its closest homologue, the lipopolysaccharide core biosynthesis protein from *Thermoplasma volcanium* GSS1 (PDB entry 3glv; Midwest Center for Structural Genomics, unpublished work), and the crystal structures of HldC has not yet been reported. Further studies are in progress to determine a full structure and to elucidate the structure–function relationship of the protein.

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